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1 A synthetic biology approach to transform *Yarrowia lipolytica* into a competitive biotechnological
2 producer of β -carotene

3

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19

20 **Competing Interests**

21 The authors declare that they have no competing interests

22 **Running title**

23 Production of β -carotene in *Y. lipolytica*

24

25 **Abstract**

26 The increasing market demands of β -carotene as colorant, antioxidant and vitamin precursor,
27 requires novel biotechnological production platforms. *Yarrowia lipolytica*, is an industrial organism
28 unable to naturally synthesize carotenoids but with the ability to produce high amounts of the
29 precursor Acetyl-CoA. We first found that a lipid overproducer strain was capable of producing more
30 β -carotene than a wild type after expressing the heterologous pathway. Thereafter, we developed a
31 combinatorial synthetic biology approach base on Golden Gate DNA assembly to screen the
32 optimum promoter-gene pairs for each transcriptional unit expressed. The best strain reached a
33 production titer of 1.5 g/L and a maximum yield of 0.048 g/g of glucose in flask. β -carotene
34 production was further increased in controlled conditions using a fed-batch fermentation. A total
35 production of β -carotene of 6.5 g/L and 90 mg/g DCW with a concomitant production of 42.6 g/L of
36 lipids was achieved. Such high titers suggest that engineered *Y. lipolytica* is a competitive producer
37 organism of β -carotene.

38

39 **Keywords**

40 *Yarrowia lipolytica*, β -carotene, metabolic engineering, synthetic biology, golden gate, promoter
41 shuffling.

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44

45 1. Introduction

46 β -carotene is an orange pigment, precursor of vitamin A. This compound is a biochemical
47 synthesized terpenoid that belongs to the group of carotenoids, together with lycopene,
48 canthaxanthin, astaxanthin among others. Carotenoids have antioxidants properties which make
49 them very relevant industrial compounds, with an expected market of \$1.4 billion in 2018 (Lin et al.
50 2014; Ye and Bhatia 2012). β -carotene is produced either chemically or biotechnologically using
51 natural producer microorganisms such as *Blakeslea trispora* (Nanou and Roukas 2016),
52 *Xanthophyllomyces dendrorhous* (Contreras et al. 2015) or *Dunaliella salina* (Wichuk et al. 2014) .
53 However, the heterologous production through metabolic engineering is considering a promising
54 way to optimize β -carotene synthesis and face the increasing market demands. Therefore, several
55 strategies to produce this compound have been carried out, mainly in model organisms such as *S.*
56 *cerevisiae* and *E. coli*. Such strategies include the expression of heterologous genes, the
57 elimination/downregulation of competing pathways, the overexpression of endogenous genes,
58 adaptive evolution, fine control of the metabolic fluxes, etc. (Lin et al. 2014; Ye and Bhatia 2012).

59 To our knowledge, the best production of β -carotene achieved in engineered baker yeast reached a
60 production of 0.45 g/L and 8.12 mg/g DCW (Xie et al. 2015). An adaptive evolution approach
61 generated the strain with the highest relative production; 18 mg/g DCW (Reyes et al. 2014).

62 Higher values have been obtained by engineering *E. coli*; 3.2 g/L from glycerol (Yang and Guo 2014)
63 or 2.47 g/L from glucose (Nam et al. 2013). One of the main drawback of using this bacteria for a
64 commercial production of β -carotene is the safety issues associated to the lack of the GRAS status.

65 *Yarrowia lipolytica* is an oleaginous yeast widely investigated and modified for the production of
66 biotechnologically relevant compounds (Ledesma-Amaro and Nicaud 2015; Liu et al. 2015; Madzak
67 2015; Zhu and Jackson 2015). It presents several advantages as industrial host, including a vast
68 repertoire of molecular tools and the ability to grow naturally in low cost substrates such as glycerol

69 or molasses (Ledesma-Amaro and Nicaud 2016), or once engineered in xylose, raw starch,
70 cellobiose, cellulose or inulin (Guo et al. 2015; Ledesma-Amaro et al. 2015; Ledesma-Amaro et al.
71 2016b; Wei et al. 2014; Zhao et al. 2010). Moreover, its metabolism, specifically the lipid
72 metabolism, has been widely studied and characterized (Dulermo et al. 2015; Dulermo et al. 2016;
73 Kerkhoven et al. 2017; Kerkhoven et al. 2016; Qiao et al. 2017; Wasylenko et al. 2015). Interestingly,
74 lipid biosynthetic pathway and carotenoid pathway share a common precursor, Acetyl-CoA, which is
75 highly available in *Y. lipolytica*. In addition, the genome of this yeast encodes all the required genes
76 to produce geranylgeranyl diphosphate (GGPP), only two compounds away from β -carotene (Figure
77 1). Because of this, *Y. lipolytica* has been recently proposed as a potential producer of carotenoids.
78 Both DuPont and Microbia have patented modified strains of *Y. lipolytica* engineered to produce
79 carotenoids (Bailey et al. 2012; Sharpe et al. 2014). Interestingly, the authors proved that modified *Y.*
80 *lipolytica* was able to produce lycopene (2mg/gDCW), β -carotene (5.7mg/gDCW), canthaxanthin and
81 astaxanthin (Sharpe et al. 2014). Another metabolic engineering approach produced 16 mg/gDCW of
82 lycopene, the direct precursor of β -carotene (Matthaus et al. 2014). Importantly, Grenfell-Lee et al.
83 have demonstrated that the safety profile of the β -carotene produced in *Y. lipolytica* is the same as
84 other commercial products, which would facilitate its commercialization (Grenfell-Lee et al. 2014).
85 Therefore, this oleaginous yeast represents a promising biotechnological chassis for the production
86 of β -carotene.

87 In addition, during the preparation of this manuscript, Gao et al. (Gao et al. 2017) engineered *Y.*
88 *lipolytica* to produce up to 4 g/L of β -carotene in fed-batch fermentation; the highest production
89 titer so far described in a heterologous microorganism. The authors engineered one strain after 12
90 steps where 11 genes were modified and they found that the integration of multiple copies of some
91 of the genes was essential to increase β -carotene production.

92 In this work we first found that a strain that overproduce lipids is more convenient for the
93 production of β -carotene than a wild type background. We further engineered the lipid

94 overproducer *Y. lipolytica* strain in order to maximize β -carotene production. For this aim, we used a
95 synthetic biology approach in order to screen the best combination of promoters for each of the
96 studied genes. Finally, we integrated the metabolic engineering of the strain with fermentation
97 condition optimization in order to boost β -carotene production, reaching the best production titer
98 and yield described so far.

99

100 **2. Material and Methods**

101 2. 1. Strains and media

102 *Escherichia coli* strain DH5 α was used for cloning and plasmid propagation. Cells were grown at 37 °C
103 with constant shaking on 5 ml LB medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl), and
104 ampicillin (100 μ g/ml) or kanamycin (50 μ g/ml) were added for plasmid selection.

105 The *Y. lipolytica* strains used in this study are derived from Po1d (wt), derived from the wild-type *Y.*
106 *lipolytica* W29 (ATCC20460) strain. All the strains used in this study are listed in Supplementary
107 Figure 1. Media and growth conditions for *Y. lipolytica* have been described elsewhere (Dulermo et
108 al. 2015). Rich media YPD and YPD60 contained 1 or 6% glucose (Sigma) respectively, 1% peptone
109 (BD bioscience) and 1% yeast extract (BD bioscience). Minimal media YNB20, YNB30 and YNB60,
110 contained 2, 3 or 6% glucose respectively (wt/vol; Sigma), 0.17% (wt/vol) Yeast Nitrogen Base
111 (YNBww; Difco), 0.5% (wt/vol) NH₄Cl and 50mM phosphate buffer (pH6.8). YNBgly60 medium was
112 prepared in the same way as the YNB medium except that 6% glycerol was added as sole carbon
113 source. When necessary, the YNB medium was supplemented with uracil (0.1 g/L) and/or leucine
114 (0.1 g/L) or hygromycin (0.2 g/L). Solid media for *E. coli* and *Y. lipolytica* was prepared by adding 15
115 g/L agar (Invitrogen) to liquid media.

116 2. 2. Strains construction

117 All restriction enzymes were purchased from New England Biolabs (NEB). PCR amplifications were
118 performed using Q5 high-fidelity DNA polymerase (NEB) or GoTaq DNA polymerase (Promega).
119 When needed PCR fragment were purified using the QIAquick Gel Extraction Kit (Qiagen). Plasmids
120 from *E. coli* were extracted using the QIAprep Spin Miniprep Kit (Qiagen). All the reactions were
121 performed according to the manufacturer instructions. Transformation of chemically competent *E.*
122 *coli* cells was performed by thermic shock protocol. Transformation of *Y. lipolytica* was performed
123 using the lithium-acetate method adapted from (Barth and Gaillardin 1996). Transformants were
124 selected on YNBLeu, YNBura, or YNBHygro media, depending on their genotype.

125 2. 3. DNA constructions

126 Primer's sequences used in this study can be found in Supplementary Table 1. The carotenoid's
127 (*GGS1-CarB-CarRP*) expression cassette (Supplementary Figure 2) named car-cassette and the t-
128 HMG's expression cassette were kindly provided by Microbia (Bailey et al. 2012) onto plasmids
129 containing the *LEU2* gene and the nourseothricin resistance gene respectively as selection markers.
130 All sequencing processes were done by Eurofins genomics. A complete plasmid list can be found in
131 Supplementary Table 2.

132 The construction of carTEF-cassette was done using the recently developed Golden Gate (GG)
133 toolbox for *Y. lipolytica* (Celinska et al. 2017) based on (Engler and Marillonnet 2014). Briefly,
134 primers carrying pre-designed 4-nt overhangs and externally located BsaI recognition sites were
135 designed and used to amplify the building blocks which were then cloned in donor vectors (Zero
136 Blunt® TOPO® PCR Cloning Kit, invitrogen). These building blocks and the destination vector carrying
137 a gene encoding for a chromophore, to facilitate selection, which is also flanked with BsaI-site and
138 pre-designed overhangs, are then mixed equimolarly (50 pmoles of ends) in one-pot reaction
139 together with 5 U of BsaI (NEB), 200 U of T4 ligase (NEB), 2 µL of T4 DNA ligase buffer (NEB) and
140 ddH₂O up to 20 µL. The following thermal profile was applied: [37°C for 5 min, 16°C for 2 min]x60,
141 55°C for 5 min, 80°C for 5 min, 15°C ∞. Subsequently, the reaction mixture was used for *E. coli*

142 transformation. White colonies were screened for identification of complete Golden Gate Assembly
143 (GGA) through plasmid isolation, restriction digestion and PCR. Complete GGA was subsequently
144 linearized by SfiI restriction enzyme and 10 μ L were used for transformation of *Y. lipolytica*.

145 All the sequences to be used as building blocks of the envisioned GG Assembly were extracted from
146 *Y. lipolytica* W29 genome sequence or from previously constructed vectors of our own collection.
147 After cloned in TOPO vector, the building blocks were screened by restriction digestion and verified
148 by sequencing.

149 A promoter shuffling was carried out to explore the combination of multiple promoters (Engler and
150 Marillonnet 2013). The assembly of plasmids with three randomized promoters (PGMp-low
151 expression, GAPDHP-medium expression, TEFp-high expression) was split into two parts: pre-
152 assembly construct and then multigene construct assembly. For the pre-assembly, three separate
153 reactions with four Golden Gate parts each (InsertionSiteUp-Marker-Promoter1-Gene1;Terminator1-
154 Promoter2-Gene2-Terminator2;Promoter3-Gene3-Terminator3-InsertionSiteDown) was done. All
155 parts were used in equimolar quantities (50 pmoles), and a mix of all three promoters was made
156 with a final concentration of 50 pmoles. Each reaction has 5 U of BsaI, 200 U of T4 ligase, 1 μ L of T4
157 DNA ligase buffer (NEB) and ddH₂O up to 10 μ L and a short thermal profile was applied: [37°C for 3
158 min, 16°C for 2 min]x30, 55°C for 5 min, 80°C for 5 min, 15°C ∞ . Subsequently, for the multigene
159 construct assembly the three previous reactions were mixed together in the same tube and the
160 destination vector (50 pmoles) was added together with 20 U of BsaI, 400 U of T4 ligase, 4 μ L of T4
161 DNA ligase buffer (NEB) and ddH₂O up to 40 μ L. The following thermal profile was applied: [37°C for
162 5 min, 16°C for 5 min]x50, 55°C for 5 min, 80°C for 5 min, 15°C ∞ . This Golden Gate reaction was
163 transformed into *E. coli*, and all transformant colonies were mixed together into a single overnight
164 culture. A plasmid library was prepared from this overnight culture, digested with SfiI restriction
165 enzyme and 10 μ L were used to transform *Y. lipolytica*. (Figure 4)

166 Identification of promoters at each site was done by PCR from gDNA of the selected yellow-orange
167 colonies obtained. For promoter in position 1 the primer pair used was Ura3Marker-intern-
168 Fw/GGSI_intern_Rv, for position 2 GGSI_intern_Fw/CarB_intern_Rv, and for position 3
169 CarB_intern_Fw/CarRP_intern_RV.

170 2. 4. β -carotene measurement

171 Intracellular β -carotene content was extracted and quantified by photometric measurement
172 adapted from previous reports (Matthaus et al. 2014). Briefly, 20 ml of medium was inoculated with
173 0.05 DO pre-culture yeast strain in a 250 ml flask. Cells were cultured during 4 days at 28°C and
174 shaking. Afterwards 200 μ l of the culture were harvested in a FastPrep FP120 (Thermo Electron) and
175 500 μ l glass beads (0.75 to 1 mm; Roth) and 1.2 ml extraction solvent (50:50 v/v; hexane-ethyl
176 acetate; 0.01% butyl hydroxyl toluene) were added. The mixture was vortexed three times for
177 1min30s at maximum speed, alternating with ice incubation. The extract was collected after 5
178 minutes centrifugation, and the extraction procedure was repeated until the pellet and the
179 supernatant were colorless. The extract was then diluted with extraction solvent and measured
180 photometrically at 448 nm. The concentrations were calculated through a standard curve using β -
181 carotene from Sigma-Aldrich as standard. The OD was correlated with the dry cell weight (DCW)
182 measurement for each corresponding culture. The washed and lyophilized cells coming from a
183 known volume served to measure the DCW. Every sample was cultured in duplicate.

184 2. 5. Microscopy images

185 Images were acquired using a Zeiss Axio Imager M2 microscope (Zeiss) with a 100 \times objective and
186 Zeiss filters 45 and 46 for fluorescent microscopy. Axiovision 4.8 software (Zeiss) was used for image
187 acquisition. Lipid bodies visualization was performed by adding BodiPy $\text{\textcircled{R}}$ Lipid Probe (2.5 mg/ml in
188 ethanol; Invitrogen) to the samples and after incubation on at room temperature for 10 min.

189 Microscopic color images were acquired using a Leica DM1000 microscope (Leica) with a 100×
190 objective a moticam 2500 camera. Motoc imaging 2.0 software was used for image acquisition.

191 2. 6. Lipid content quantification

192 Lipids were extracted from 15–25 mg aliquots of lyophilized cells and converted into their equivalent
193 methyl esters as previously described (Ledesma-Amaro et al. 2016a). The products were then used
194 in the gas chromatography (GC) analysis, performed using a Varian 3900 gas chromatograph
195 equipped with a flame ionization detector and a Varian FactorFour vf-23 ms column, where the
196 bleed specification at 260 °C was 3 pA (30 m, 0.25 mm, 0.25 µm). FA were identified by comparison
197 to commercial fatty acid methyl ester standards (FAME32, Supelco) and quantified by the internal
198 standard method with the addition of 50 µg of commercial C12:0 (Sigma).

199 The washed and lyophilized cells coming from a known volume served to measure the DCW.

200 2. 7. Biomass, sugar and acid quantification

201 Dry cell weight (DCW) was calculated by weighting the lyophilized cells. The harvested cells were
202 washed twice and centrifuged in order to separate all the mass remaining in the culture media prior
203 to lyophilisation.

204 Sugar and citric acid were quantified by HPLC (UltiMate 3000, Dionex-Thermo Fisher Scientific, UK)
205 using an Aminex HPX 87 H column coupled to UV (210 nm) and RI detectors. The column was eluted
206 with 0.01 N H₂SO₄ at room temperature and a flow rate of 0.6 ml min⁻¹. Identification and
207 quantification were achieved via comparisons to standards. Before being subject to HPLC analysis,
208 samples were filtered on 0.45-µm pore-size membranes.

209 2. 8. Bioreactor procedures

210 Fed-batch cultivations were performed in a 5 L bioreactor (Sartorius Stedim Biotech, Göttingen,
211 Germany) with an initial working volume of 2 L. The initial medium (Y10P20D) contained 10 g/L of

212 Yeast extract (Becton Dickinson, Franklin Lakes, USA), 20 g/L of peptone (Becton Dickinson, Franklin
213 Lakes, USA) and 5 g/L of glucose (Sigma-Aldrich, St. Louis, USA). The concentrated medium
214 (Y20P40D) contained 20 g/L of Yeast extract, 40 g/L of peptone and 5 g/L of glucose. The
215 temperature was held constant at 28°C, the aeration at 2 VVM, the agitation at 500 - 900 rpm, the
216 pH at 5.5 automatically by injection of 100 g/L H₃PO₄ or 200 g/L KOH, and the dissolved oxygen was
217 set up at 20 %. Foam was prevented by the addition of antifoam 204 (Sigma-Aldrich, St. Louis, USA).
218 The medium was inoculated with 100 mL from a 24 h preculture performed in a shake flask
219 containing 10 g/L of Yeast extract, 20 g/L of peptone and 20 g/L of glucose. The fed-batch process
220 was initiated after 6 h of cultivation at a rate of 6 g/L (0.2 molcarbon/h) of glucose from a 500 g/L
221 concentrated stock solution.

222

223 **3. Results and Discussion**

224 3. 1. Lipid overproducer strain synthesizes higher amount of β -carotene

225 Previous reports have proven that the overexpression of three genes, geranylgeranyl diphosphate
226 synthase (*GGG1* from *Y. lipolytica*), phytoene synthase/lycopene cyclase and phytoene dehydrogenase
227 (*carPR* and *carB* from *Mucor circinelloides*) promote β -carotene production in *Y. lipolytica* (Celinska
228 et al. 2017; Gao et al. 2014). We here used an expression cassette (*car*-cassette) where the
229 expression of *GGG1* is controlled by the promoter PGMp, of *CarB* by GAPDHp and of *CarRP* by TEF1p.
230 As expected, the sole expression of this cassette in the parental strain (named wt-C) allows it to
231 produce substantial amounts of β -carotene (3.4 mg/gDCW and 18.4 mg/L) (Figure 2). This
232 production level was higher than the 2.2 mg/gDCW previously obtained for a similar approach (Gao
233 et al. 2014). The differences could be caused by the use of different promoters controlling each of
234 the three genes or due to the use of different parental strains.

235 It is well known that the lipophilic nature of carotenoids promotes their storage in the lipid bodies of
236 the cells. In Supplementary Figure 3 the co-localization of the pigment and the neutral lipids can be
237 seen. Here we hypothesized that lipid overproducer strains could boost both β -carotene production,
238 due to a higher availability of the precursor Acetyl-CoA, and storage, due to an increase of the
239 lipophilic structures inside the cells. In order to verify this, we transformed a lipid overproducer
240 strain (JMY3501, hereafter called 'obese' or 'ob') with the car-cassette. As expected, the generated
241 strain (ob-C) accumulated higher amount of lipids, 3.6 times more than the strain wt-car, and
242 interestingly it also boosted β -carotene synthesis, producing 8.9 mg/gDCW and 35.7 mg/L, 2.61 and
243 1.93 times more than wt-car (Figure 2). The higher amounts of lipids could be important for the
244 solubilisation of β -carotene, which would reduce the formation of crystals that could impair the
245 cellular homeostasis. These results indicate the importance of selecting the proper parental strain in
246 metabolic engineering approaches. Unexpectedly, the expression of the β -carotene pathway
247 favoured the total production of lipids, which increased 82% and 56% in the wild type and obese
248 background respectively. Importantly, the co-production of the two biotechnologically relevant
249 compounds, β -carotene and lipids, could facilitate the industrial viability of the process.

250

251 3. 2. Further metabolic engineering increases carotene content and reveals pathway bottlenecks

252 The overexpression of hydroxymethylglutaryl-CoA reductase (*HMG1*) is known to channelized the flux
253 towards carotenoids in engineered microorganisms (Matthaus et al. 2014). Here we overexpressed
254 a truncated version of this gene (YALI0E04807,(Bailey et al. 2012)) under the control of the
255 constitutive TEF promoter in the strain ob-C. The generated strain (ob-CH) increased β -carotene
256 content up to 17.4 mg/gDCW and 121.6 mg/L, 2.0 and 3.4 times more than the parental strain ob-C
257 (Figure 3). Again, this results are higher than the ones recently obtained from a strain overexpressing
258 the same genes but using different parental strain and set of promoters (4.36 mg/gDCW and 64.6
259 mg/L)(Gao et al. 2017).

260 In order to analyze if the production of β -carotene is limited by the expression levels of the
261 overexpressed genes we introduced in the genome of ob-CH a second copy of the car-cassette,
262 generating the ob-CHC. Interestingly, we found a further increase in the desired product, which
263 reached 24.0 mg/gDCW and 175.6 mg/L, 1.4 and 1.4 times more than the parental strain ob-CH. This
264 result indicates that the expression of some of the gene encoded in the car-cassette is limiting the
265 production of β -carotene. Similar conclusion was reached by Gao et al. (Gao et al. 2017) in a parallel
266 study where the authors improved the β -carotene content by increasing the copy number of the
267 genes expressed. Here, in order to overcome this limitation we considered a strategy that combines
268 the increase of the copy number and the optimization of the promoter strength for each gene.

269

270 3.3. Promoter shuffling using Golden Gate indentifies the best promoter set for the production of β -
271 carotene

272 We have previously seen (Dulermo et al. 2017) that the increase in the promoter strength, even in
273 strong promoters, can enhance the transcription level more than 6 times, while an extra copy of a
274 gene under the same promoter can typically only duplicate the expression level. We therefore
275 decided to identify the best combination of promoters and genes in order to maximize β -carotene
276 production. The construction of the car-cassette with different promoters via traditional cloning
277 would have been a very long and inefficient process. Thus, we took advantage of the recently
278 developed Golden Gate toolbox for *Y. lipolytica* (Celinska et al. 2017) to perform a promoter
279 shuffling strategy. The strategy, summarized in Figure 4, consists in a digestion-ligation reaction
280 guided by BsaI defined sites where the three promoters can be introduced in each of the three
281 promoter positions in the car-cassette. It has been recently proven by sequencing the Golden Gate
282 reaction products that every possible combination is unbiased generated during combinatorial
283 assemblies (Awan et al. 2017). Then, the pool of cassettes were simultaneously amplified, linearized
284 and used to transform the wild type parental strain (wt), where the difference in colour intensity

285 could be easily screened (Supplementary Figure 4). After the transformation, 387 colonies with
286 different colour were obtained. Here, we selected the 15 more orange-yellow strains and we
287 checked the set of promoter controlling each gene (Figure 5). Interestingly, the combination of
288 promoters originally present in the car-cassette was not found among the best producer strains and,
289 accordingly, the analyzed strains generated by the shuffling produced higher β -carotene content
290 than the wt-car previously analyzed (Figure 2). The major conclusion of these results is that the
291 cassette with the three genes controlled by TEF1p is the optimum producer. Analyzing the overall
292 combination of promoters found as convenient for overproducing β -carotene, it seems that the
293 presence of a strong TEF promoter is favoured in the second position of the car-cassette (11 out of
294 15). The best of these strains were able to produce 6.3 times more β -carotene than the wt-car, 16.7
295 mg/gDCW and 111.8 mg/L.

296 Based on these results we constructed using Golden Gate a new car-cassette where the three genes,
297 *GGs1*, *CarB* and *CarRP* are under the control of TEF1 promoter. This new cassette was called car^{TEF}-
298 cassette and it was used to further increase the β -carotene content of ob-CH.

299

300 3. 4. Construction of a β -carotone overproducer strain

301 We have previously seen that an extra copy of the car-cassette in the ob-CH increased β -carotene
302 production and, in addition, we have improved the cassette (car^{TEF}-cassette) by an optimized set of
303 promoters. We therefore attempted to further increase total β -carotene content by the expression
304 of car^{TEF}-cassette in the strain ob-CH. The generated strain, ob-CHC^{TEF}, as hypothesized, produced
305 higher β -carotone; 54.4 mg/gDCW and 293.3 mg/L (Figure 6).

306 Finally, an extra copy of the car^{TEF}-cassette was introduced to construct the strain ob-CHC^{TEF}C^{TEF}. This
307 strain was able to produce 61.1 mg/gDCW and 454.36 mg/L of β -carotene (Figure 6), which
308 represent the best production of this compound so far described in flask culture (260 mg/L in *E. coli*

309 (Yang and Guo 2014) and 353.6 mg/L in *Y. lipolytica* (Gao et al. 2017) and the best yield reported so
310 far (0.048 g/g followed by 0.027 g/g achieved in *E. coli* (Yang and Guo 2014) and 0.018 g/g in *Y.*
311 *lipolytica* (Gao et al. 2017)). We also tested the stability of this strain, since some reports in *S.*
312 *cerevisiae* showed the appearance of spontaneous white colonies (Beekwilder et al. 2014) but in *Y.*
313 *lipolytica* all the cells plated after 6 days of culture produced β -carotene (Supplementary Figure 5),
314 which could be expected from the low homologous recombination rate in this yeast. We therefore
315 selected this strain in order to optimize culture conditions to further increase β -carotene production.

316

317 3. 5. Media optimization shows a trade-off between production titer and yield

318 In order to study the effect of the media composition in the production of β -carotene, we tested two
319 different kind of media, rich media (YPD) and synthetic media (YNB). We also tested different
320 concentrations of carbon source keeping constant the nitrogen amount, referred by a number that
321 indicates the concentration in g/L (10, 20, 30 and 60). It is well known that a higher C/N ratio
322 promotes lipid production as well as other carbon based molecules such as carotenoids (Braunwald
323 et al. 2013). We tested glucose as a standard carbon source and glycerol (GLY) as a cheaper carbon
324 sources. The selected media were YPD10, YPD60, YNB20, YNB30, YNB60 and YNBGLY60. Large
325 variations in the β -carotene production were found depending on the culture media (Figure 7).
326 According to the results we can suggest that there is no much influence on the carbon source since
327 glucose and glycerol showed similar titer and yields. In addition, a clear correlation between the
328 increase in the initial glucose content and the production titer was found in both rich and synthetic
329 media. Interestingly, the production yields for all the YNB based-media tested was similar
330 independently of the amount or kind of carbon source. However, this was not the case for rich
331 media where a trade-off between production titer and yield was found. The best β -carotene titer so
332 far, 1.5 g/L, was found in YPD60 while the best yield, 0.048 g/g was produced in YPD10. In any case,

333 YPD showed higher titers and yields than YNB and therefore we selected rich media for further
334 optimization of the culture conditions in a controlled fermentation in bioreactor.

335

336 3.6. Bioreactor controlled conditions boosts β -carotene production by the engineered strain ob-
337 CHC^{TEF}C^{TEF}

338 In order to improve the production of β -carotene in the strain ob-CHC^{TEF}C^{TEF} we decided to optimize
339 the culture conditions using a 5 L bioreactor. We here performed a fed-batch fermentation using rich
340 media (Y10P20D) where glucose was added after 6 h of cultivation at a rate of 6 g/h. The
341 fermentation was stopped after 143.5 h when the glucose concentration started to rise in the
342 culture media indicating its lack of consumption. The strain reached a production 2.9 g/L β -carotene
343 and a concomitant production of 40 g/L lipids and 31 g/L of citric acid (Supplementary Figure 6).

344 In order to further optimize bioreactor conditions we designed a culture media with double amounts
345 of yeast extract and peptone, named Y20P40D, where the rate of glucose feeding was used as
346 carbon source in fed-batch. In this experiment, a maximum production of 6.5 g/L of β -carotene was
347 produced after 122 hours, the best titer described so far (Figure 8). In this conditions, the production
348 of citric acid was kept under 1 g/L along the fermentation while the lipid titer reached 40 g/L (Figure
349 8). Interestingly, the maximum β -carotene content was 89.6 mg/g of DCW, 50 % higher than in flask
350 and one of the highest described in the literature. Such results indicates that herein engineered *Y.*
351 *lipolytica* using synthetic biology and metabolic engineering is a potential industrial producer of β -
352 carotene.

353

354 **4. Conclusions**

355 In this work we have combined traditional metabolic engineering strategies with novel synthetic
356 biology tools in order to turn *Y. lipolytica* an industrially competitive producer of β -carotene. We
357 found that increasing lipogenesis and gene copy number as well as using the most favourable set of
358 promoters, greatly enhanced β -carotene production. Finally a fed-batch fermentation lead to the
359 highest β -carotene production reported so far.

360 This work shows the enormous potential of *Y. lipolytica* to produce β -carotene in an economically
361 feasible manner, not only by the high titer achieved but also due to the co-production of high
362 amount of lipids, which can be used as fuels or chemicals (Singh et al. 2016). Moreover, the process
363 can be further improved not only by strain engineering and bioreactor condition optimization but
364 also by the use of low cost carbon sources such as starch or lignocellulosic materials. In addition, this
365 work also highlights the importance of selecting the right microorganism and strain in a synthetic
366 biology or metabolic engineering strategy. The selection of an oleaginous organism has permitted
367 production levels far beyond the so far obtained in other yeasts such as *S. cerevisiae*, while the
368 selection of a lipid overproducer strain further increased β -carotene content. This work is an
369 example of how the rapid development of synthetic biology tools for DNA assembly and genome
370 editing is facilitating the manipulation of non-conventional organisms, expanding the range of
371 biotechnological chassis for metabolic engineering.

372

373 **Acknowledgments**

374 We would like to thanks Microbia for providing the car-cassette and the HMG cassette.

375

376

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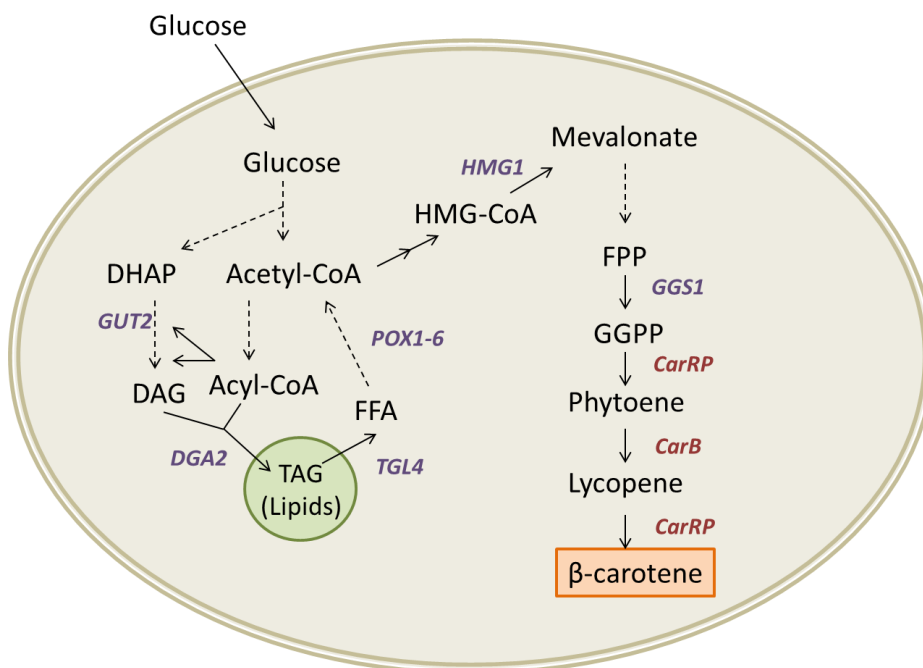
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481

482

483 **Figures**

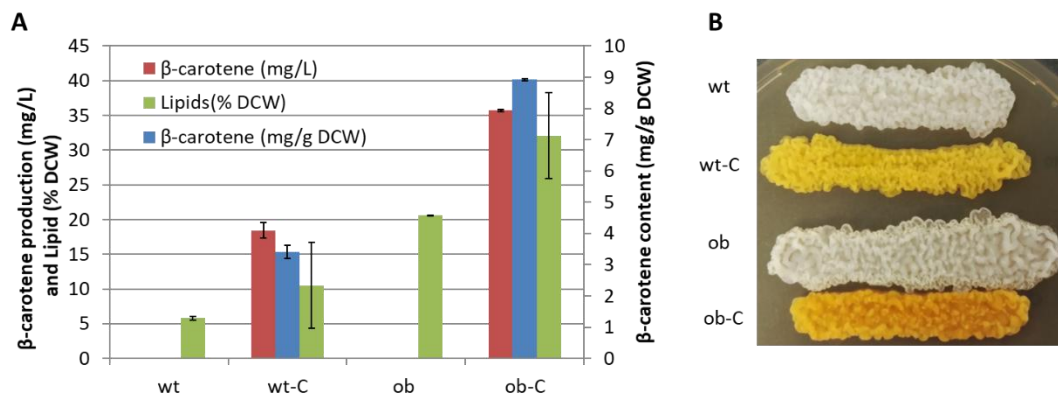


484 Figure 1: Scheme of the metabolic pathways leading to the production of lipids and β-carotene in *Y.*
 485 *lipolytica*.

486 Metabolites are shown in black, native enzymes in purple and heterologous enzymes in red. The
 487 continuous arrows indicate a metabolic step while the dashed arrows indicate multiple metabolic
 488 steps. DHAP, dihydroxyacetone phosphate; DAG, diacylglycerol; TAG, triacylglycerol; FFA, free fatty
 489 acids; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

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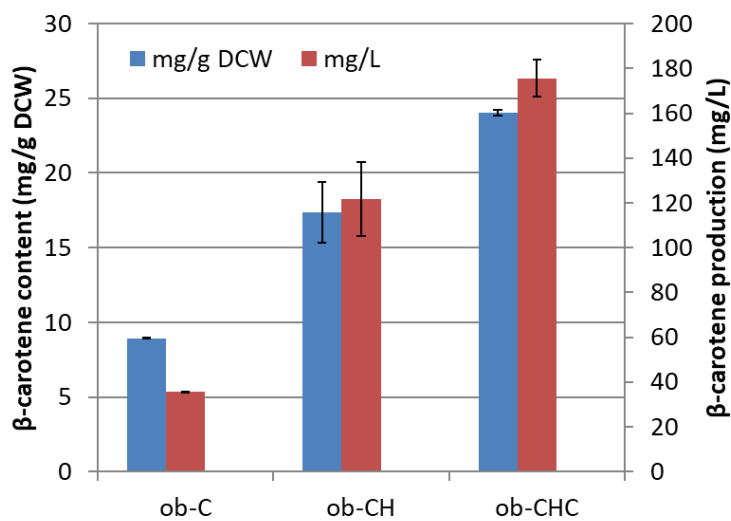
491



492 Figure 2: Lipids and β -carotene production in wt and ob backgrounds.

493 A) β -carotene production (mg/L), content (mg/g DCW) and lipid content (% DCW) in the strains wt,
 494 wt-C, ob and ob-C after growing in YPD for 4 days. The average value and the SD from two
 495 independent experiments are shown. B) Strains wt, wt-C, ob and ob-C growing on YPD-agar plate
 496 where the color provoked by the β -carotene can be seen.

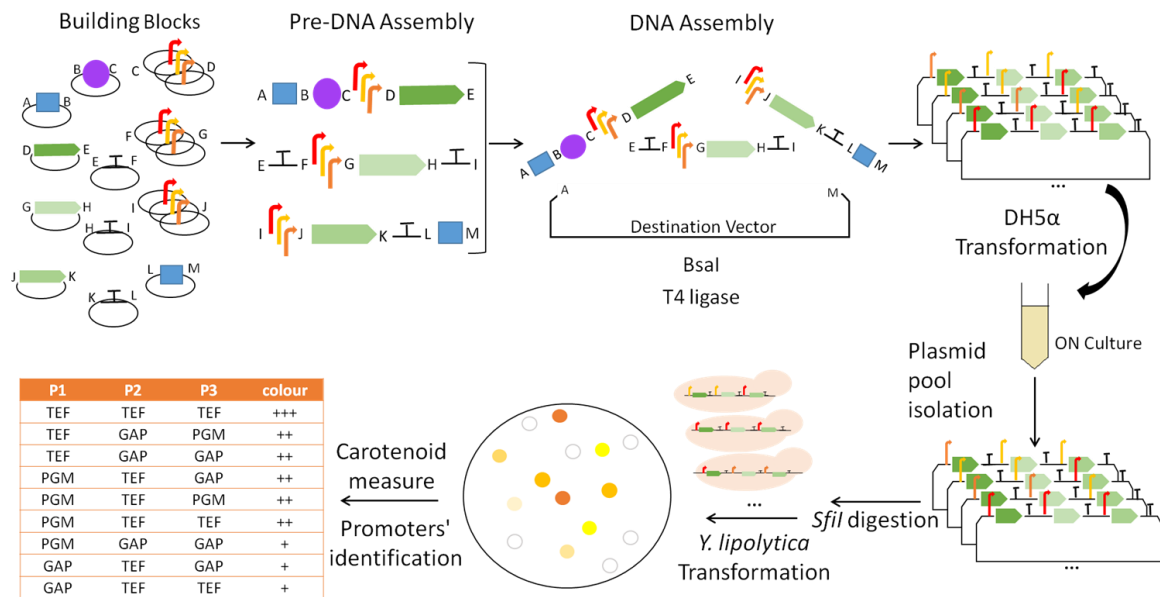
497



498 Figure 3: β -carotene production in ob-derived background.

499 A) β -carotene production (mg/L) and content (mg/g DCW) in the strains ob-C, ob-CH and ob-CHC
 500 after growing in YPD for 4 days. The average value and the SD from two independent experiments
 501 are shown.

502

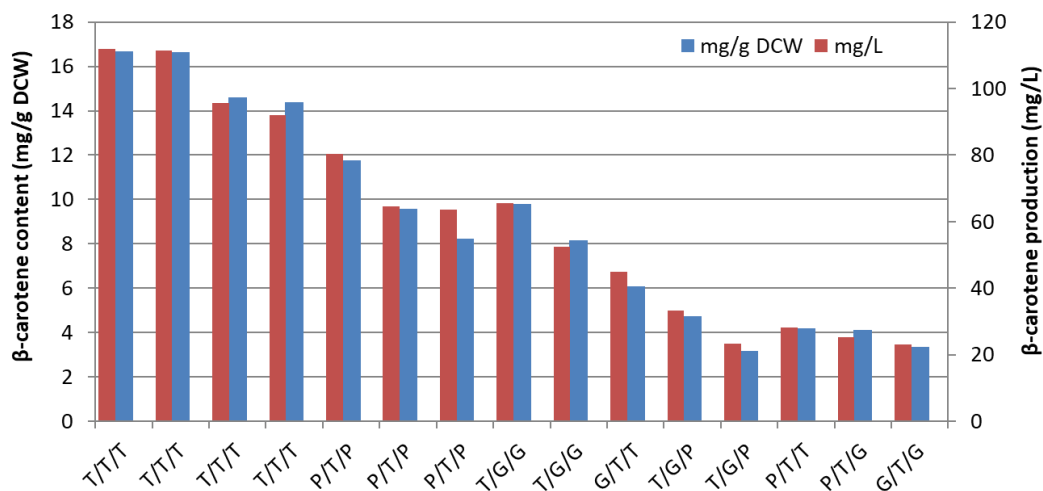


503

504 Figure 4: Scheme of the promoter shuffling strategy to optimize β -carotene production in *Y.*
505 *lipolytica*.

506 Briefly, Golden Gate cloning system was used to generate a pool of randomized expression cassettes
507 bearing different set of 3 different promoters. The pool of expression cassettes was used to
508 transform *Y. lipolytica* and the generated clones were screened by colour intensity. The selected
509 clones were further analyzed, each promoter set was determined and the β -carotene content was
510 measured (see Materials and methods for a detailed explanation). Letters A to M represent the
511 designed overhangs enabling the ordered assembly of DNA parts after Bsal digestion. Blue squares
512 represent genomic integration targeting sequences, violet circle represents the selection marker
513 gene, red-orange-yellow arrows represent promoters, green arrows represent genes and T represent
514 terminators.

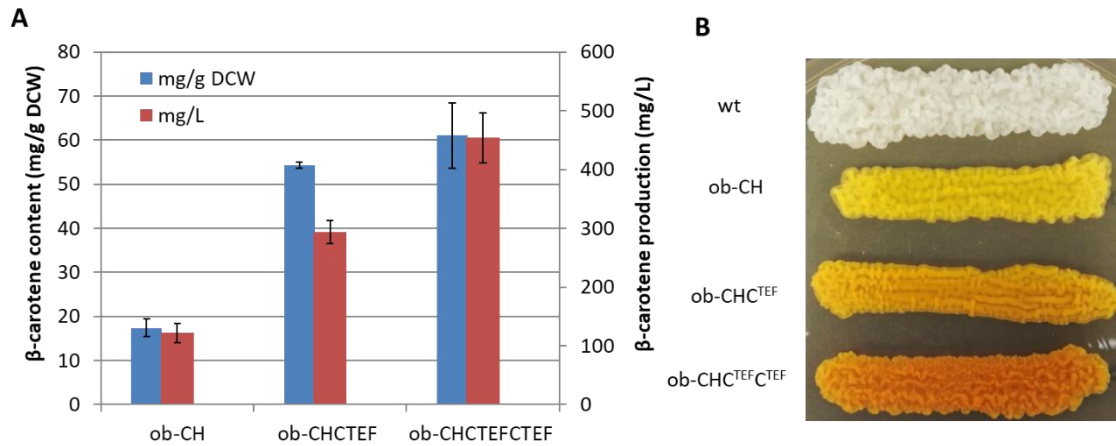
515



516 Figure 5: β -carotene production in the strains selected from the promoter shuffling approach.

517 β -carotene production (mg/L) and content (mg/g DCW) in the strains derived from the promoter
 518 shuffling experiment after growing in YPD for 4 days. Each strain is identified by the set of
 519 promoters: T, TEFp; P, PGMp; G, GAPDHp in the car-cassette order 1st GGS1, 2nd carB and 3rd carRP.
 520 Some of the selected strains presented the same combination of promoters.

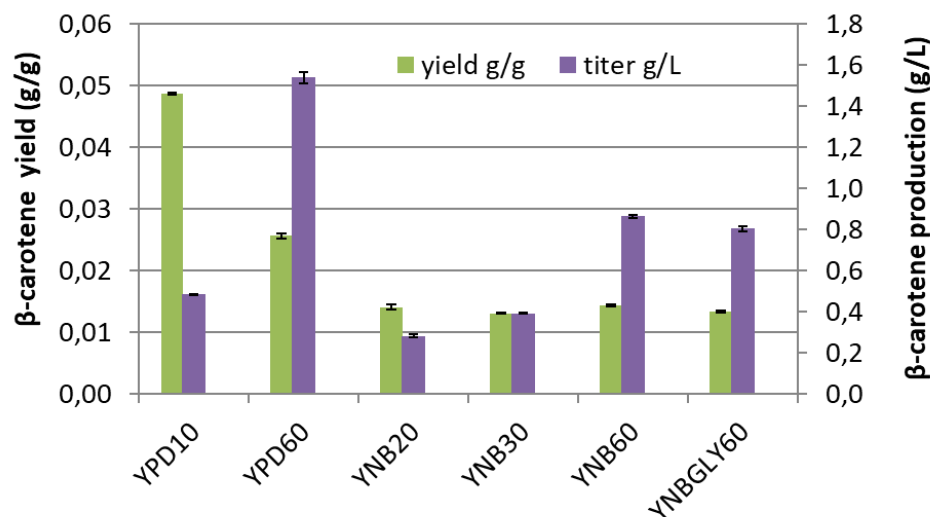
521



522 Figure 6: β -carotene production in engineered strains of *Y. lipolytica*.

523 A) β -carotene production (mg/L) and content (mg/g DCW) in the strains ob-CH, ob-CHCTEF and ob-
 524 CHCTEFCTEF after growing in YPD for 4 days. The average value and the SD from two independent
 525 experiments are shown. B) Strains wt, ob-CH, ob-CHCTEF and ob-CHCTEFCTEF growing on YPD-agar
 526 plate where the color provoked by the β -carotene can be seen.

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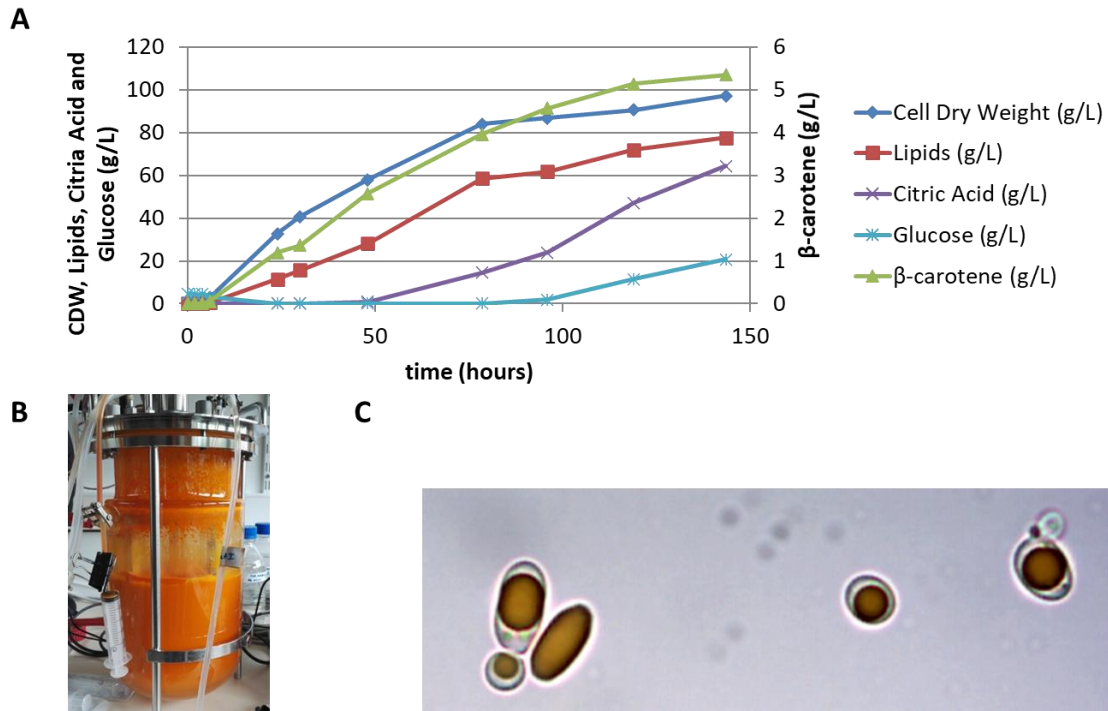


528 Figure 7: Yield and titer of β -carotene production in different culture media.

529 A) β -carotene yield (g β -carotene/g substrate) and titer (g β -carotene/L) in the strain ob-CHCTEFCTEF
 530 after growing for 4 days in rich media (YPD10, 10g/L glucose or YPD60, 60g/L glucose) or synthetic

531 media (YNB20, 20g/L glucose, YNB30, 30g/L glucose, YNB 60, 60 g/L glucose or YNBGLY60, 60 g/L
 532 glycerol). The average value and the SD from two independent experiments are shown.

533

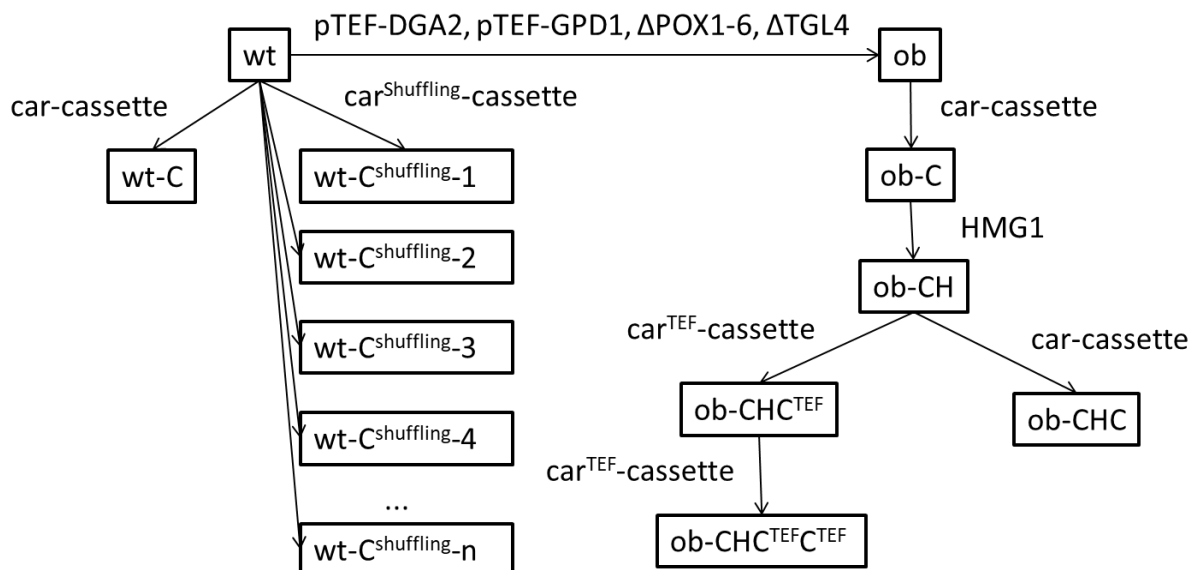


534 Figure 8. Production of β -carotene by engineered *Y. lipolytica* in bioreactor in Y20P40D media.

535 A) Kinetics of the 5-L bioreactor fermentation along 143.5 hours showing Cell Dry Weight (g/L), lipids
 536 (g/L), citric acid (g/L), glucose (g/L), β -carotene (g/L) and bioreactor broth volume (L). B) Picture of
 537 the bioreactor after 120 hours in Y10P20D. C) microscopic image of the cells grown in Y10P20D
 538 where the β -carotene can be seen as the orange colour staining the lipid bodies of the cells.

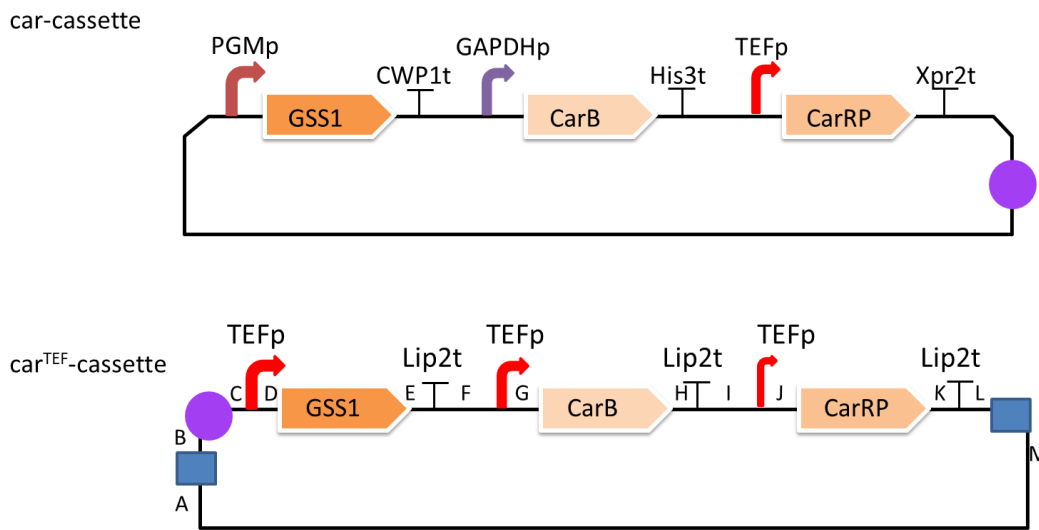
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541 Supplementary Figure 1. Scheme showing the strains constructed and used in this study.

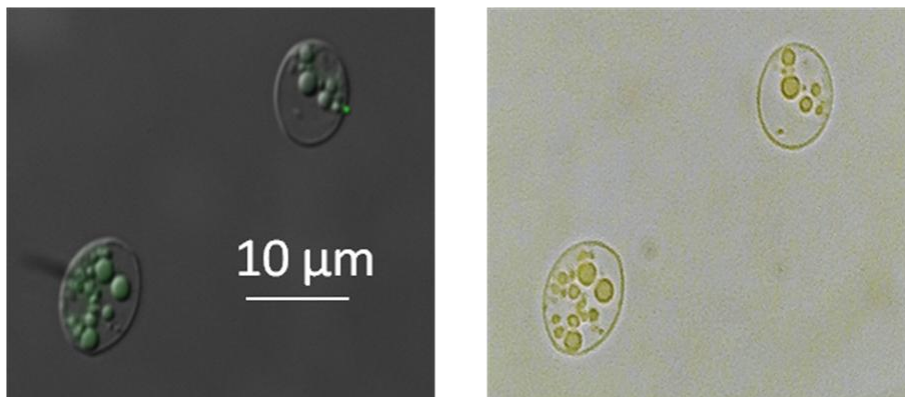
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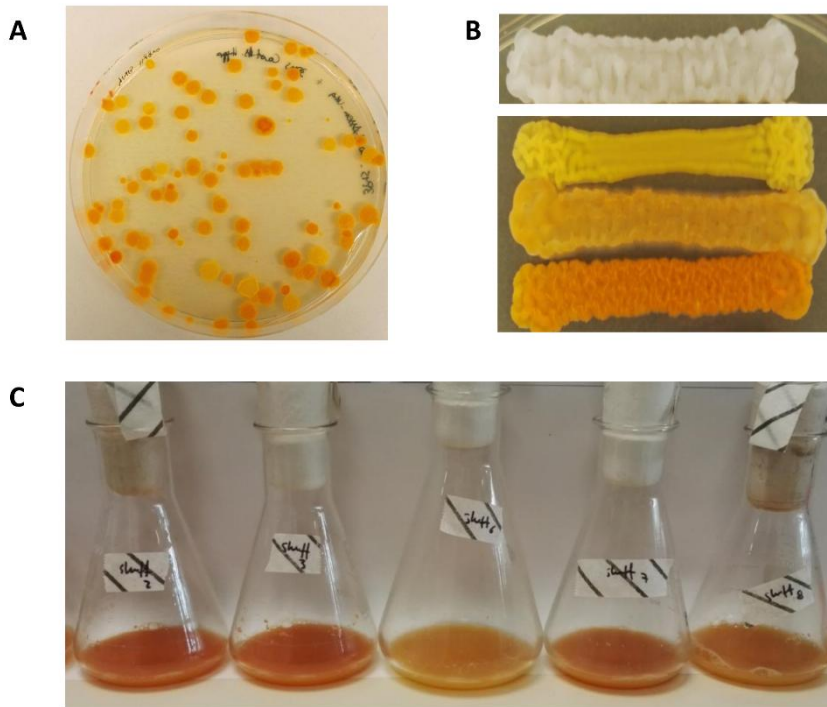
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544 Supplementary Figure 2. Schematic representation of the car-cassettes used in this study.

545

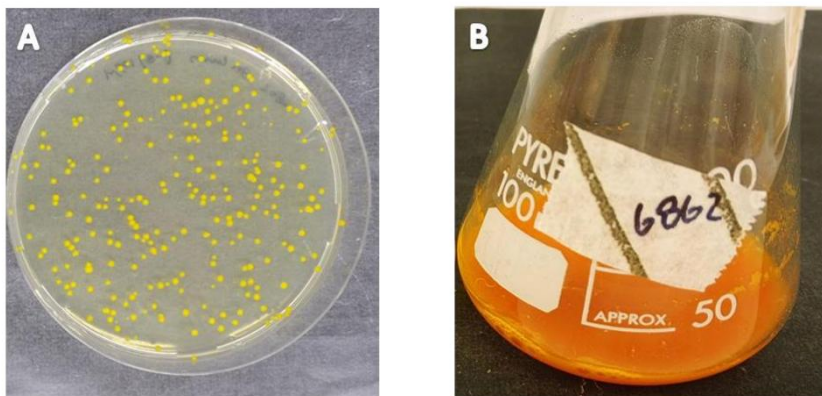


546 Supplementary Figure 3: co-localization of lipid bodies (left, fluorescence microscopy stained with
547 bodipy) and β-carotene (right, light microscopy revealing the orange β-carotene).

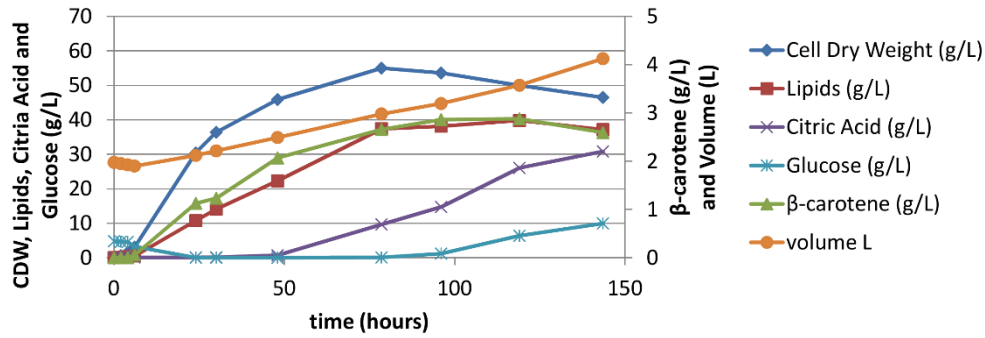


549 Supplementary Figure 4. Example of colour variation in the shuffling promoter experiment after
550 plating the transformation (A) and growing some of the selected strain in YPD solid (B) or liquid (C)
551 for 4 days.

552



553 Supplementary Figure 5. Stability test. The engineered strain ob-CHCTEFCTEF was plated (A) from a 6
554 days culture in YPD (B).



555

556 Supplementary Figure 6. Production of β -carotene by engineered *Y. lipolytica* in bioreactor in
 557 Y10P20D media.

558 Kinetics of the 5-L bioreactor fermentation along 143.5 hours showing Cell Dry Weight (g/L), lipids
 559 (g/L), citric acid (g/L), glucose (g/L), β -carotene (g/L) and bioreactor broth volume (L).

560

561 **Tables**

562 Supplementary Table 1. Oligonucleotide table. The nucleotides in capital letters show the BsaI
 563 recognition site and the 4 nt overhangs for the Golden Gate strategy.

OLIGONUCLEOTIDES	
Name	Sequence
Amplification of GG Parts	
GGP_InsertUP_zeta_A_F_Sfil	GGTCTCtGCCTggccacctaggcctgtcgggaaccgc
GGP_InsertUP_zeta_B_R	GGTCTCTACCTtctagcaaagtgtttgtgc
GGP_M_Ura3_B_F	GGTCTCTAGGTataaactcgtatagcatacattatacgaag
GGP_M_Ura3_C_R_new	GGTCTCtCCGTtcgcttcggataactcc
GGP_M_Hygro.2.1_B_F	gggGGTCTCTAGGTgcatacattatacgaagtattctg
GGP_M_Hygro.2.1_C_R_new	ggGGTCTCTCCGTatacgaattctcgactattcctttg
GGP_P1_TEF_C_F_New	GGTCTCtACGGgggttgccggcg
GGP_P1_TEF_D_R	GGTCTCTCATTtctcgggtgtgagttgac
GGP_P1_PGM_C_F_New	GGTCTCtACGGtaccacacagattacgac
GGP_P1_PGM_D_R	GGTCTCTCATTttttgtatgtgttttggtgatgtc
GGP_P1_GAPdh_C_F_new	GGTCTCtACGGcggtagtcggaagagc
GGP_P1_GAPdh_D_R	GGTCTCTCATTtgttgatgtgtttaattcaagaatg
GGP_G1_GGS1_D_F	GGTCTCTAATGgattataacagcgcgg
GGP_G1_GGS1_E_R	GGTCTCTTAGAtcactgcgcatcctc
GGP_T1_LIP2inclT_E_F	GGTCTCTTCTAggtctgtggtatctaagctattatcactctttacaacttctacctcaactatctactttaataaatgaatcgc
GGP_T1_LIP2inclT_F_R	GGTCTCTAAGCtcttagaggaacgcatacagtaatcatagagataaaacgatattcatttataaagtagatagttgaggtagaagttg
GGP_P2_TEF_DSM_F_F	GGTCTCTGCTTgacgggttgccgg
GGP_P2_TEF_DSM_G_R	GGTCTCTTGTgattcttataactcagaaggaaatgc
GGP_P2_PGM_F_F	GGTCTCTGCTTtaccacacagattacgac
GGP_P2_PGM_G_R	GGTCTCTTGTttttgtatgtgttttggtgatgtc
GGP_P2_GAPdh_F_F	GGTCTCTGCTTcggtagtcggaagagc
GGP_P2_GAPdh_G_R	GGTCTCTTGTtgttgatgtgtttaattcaagaatg
GGP_G2_carB_G_F	GGTCTCTACAAtgtccaagaaacacattgtcattatc
GGP_G2_carB_H_R	GGTCTCTATCCttaatgacattagagttatgaacgc
GGP_T2_LIP2inclT_H_F	GGTCTCTGGATgtgtctgtggtatctaagctattatcactctttacaacttctacctcaactatctactttaataaatgaatcgc
GGP_T2_LIP2inclT_I_R	GGTCTCTTGACTgtcttagaggaacgcatacagtaatcatagaga

	ataaacgatattcatttattaaagtagatagttgaggtagaagttg
GGP_P3_TEF_DSM_I_F	GGTCTCTGTCAgacgggttggcgg
GGP_P3_TEF_DSM_J_R	GGTCTCTGTGGtgattcttatactcagaaggaatgc
GGP_P3_PGM_I_F	GGTCTCTGTCAaccaaccacagattacgac
GGP_P3_PGM_J_R	GGTCTCTGTGGttttgatgtgttttggtgatgct
GGP_P3_GAPdh_I_F	GGTCTCTGTCAcggtagtcggaagagc
GGP_P3_GAPdh_J_R	GGTCTCTGTGGgttgatgtgttttaattcaagaatg
GGP_G3_carRP_J_F	GGTCTCTCCACAatgctgctcacctacatg
GGP_G3_carRP_K_R	GGTCTCTGTATttaaaggatttagatttctcattttccc
GGP_T3_LIP2inclT_K_F_NEW	GGTCTCTGTATgtgtctgtggtatctaagctattatcatctttacaacttctacacactatctacttaataaatgaatatcg
GGP_T3_LIP2inclT_L_R	GGTCTCTACTCgtcttagaggaacgcatatacagtaatcatagagaataaacgatattcatttattaaagtagatagttgaggtagaagttg
GGP_InsertDOWN_zeta_L_F	GGTCTCTGAGTcatgtgtaacctcgcctcg
GGP_InsertDOWN_zeta_M_R_Sfil	GGTCTCTCGCAggcctcctggcactgaaggccttgtgag
Elimination of internal BsaI site through assembly PCR	
GGS1_Bsa_del_F	gaaatagtctcgagatcgctcttg
GGS1_Bsa_del_R	gagactatttcgcacatcaccaag
Verification of constructions	
ZetaUp-Intern-Fw	tatctctgacgcattgaccac
Ura3Marker-intern-Rv	caactaactcgtactattacc
Ura3Marker-intern-Fw	acatccagagaagcacacagg
GGS1_intern_Rv	cactgcatcctcaaagta
GGS1_intern_Fw	tcaaggagatatggggcaag
CarB_intern_Rv	ccattggattgaggaagaa
CarB_intern_Fw	atgccaccaacaagtgaca
CarRP_intern_RV	tgtgctctgctgggatagtg
CarRP_intern_Fw	cccttctgttaccgaccaa
ZetaDown-Intern-Rv	ggtaacgccgattctctctg

564

565 Supplementary Table 2: Plasmid table

PLASMIDS		
Name	Resistance/Marker	Function (reporter)
pCR™-Blunt II-TOPO®	Kanamycin	GG Donor vector
GGV TOPO InsUp-Zeta	Kanamycin	GG building block storage Insertion sequence 5' Zeta
GGV TOPO M-URA	Kanamycin	GG building block storage. Marker URA3
GGV TOPO M-Hygro	Kanamycin	GG building block storage. Marker Hygromycin Resistance
GGV TOPO P1-TEF	Kanamycin	GG building block storage. Promoter 1 st position TEF
GGV TOPO P1-PGM	Kanamycin	GG building block storage. Promoter 1 st position PGM
GGV TOPO P1-GAPdh	Kanamycin	GG building block storage. Promoter 1 st position GAPdh
GGV TOPO G1-GSS1	Kanamycin	GG building block storage. Gene 1 st position GSS1
GGV TOPO T1-Lip2	Kanamycin	GG building block storage. Terminator 1 st position Lips2
GGV TOPO P2-TEF	Kanamycin	GG building block storage. Promoter 2 nd position TEF
GGV TOPO P2-PGM	Kanamycin	GG building block storage. Promoter 2 nd position PGM
GGV TOPO P2-GAPdh	Kanamycin	GG building block storage. Promoter 2 nd position GAPdh
GGV TOPO G2-CarB	Kanamycin	GG building block storage. Gene 2 nd position GSS1
GGV TOPO T2-Lip2	Kanamycin	GG building block storage. Terminator 2 nd position Lips2
GGV TOPO P2-TEF	Kanamycin	GG building block storage. Promoter 3 rd position TEF
GGV TOPO P2-PGM	Kanamycin	GG building block storage. Promoter 3 rd position PGM
GGV TOPO P2-GAPdh	Kanamycin	GG building block storage. Promoter 3 rd position GAPdh
GGV TOPO G3-CarRP	Kanamycin	GG building block storage. Gene 3 rd position GSS1
GGV TOPO T3-Lip2	Kanamycin	GG building block storage. Terminator 3 rd position Lips2
GGV TOPO InsD-Zeta	Kanamycin	GG building block storage Insertion sequence 3' Zeta
pSB1A3-GB3	Ampicilin	GG Destination vector (GB3 – red chromophore)
GGAV 3TEF-Carotenoids-URA	Ampicilin/ URA3	GG Assembly expression cassette 3TEF_GSS1-CarB-CarRP-

		<u>_URA3</u>
GGAV 3TEF-Carotenoids-Hygro	Ampicilin/ Hygromycin	GG Assembly expression cassette 3TEF_GSS1-CarB-CarRP_Hygromycin
pMB6511	Ampicilin / Leu2	Carotenoid expression cassette
pMB6205	Kanamycin/ Hygromycin	HMG expression cassette

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